K63-Linked Ubiquitination of GABAB1 at Multiple Sites by the E3 Ligase Mind Bomb-2 Targets GABAB Receptors to Lysosomal Degradation

Zemoura, Khaled ; Trümpler, Claudia ; Benke, Dietmar

Abstract: GABAB receptors are heterodimeric G protein-coupled receptors, which control neuronal excitability by mediating prolonged inhibition. The magnitude of GABAB receptor-mediated inhibition essentially depends on the amount of receptors in the plasma membrane. However, the factors regulating cell surface expression of GABAB receptors are poorly characterized. Cell surface GABAB receptors are constitutively internalized and either recycled to the plasma membrane or degraded in lysosomes. The signal that sorts GABAB receptors to lysosomes is currently unknown. Here we show that Mind bomb-2 (MIB2) mediated K63-linked ubiquitination of the GABAB1subunit at multiple sites is the lysosomal sorting signal for GABAB receptors. We found that inhibition of lysosomal activity in cultured rat cortical neurons increased the fraction of K63-linked ubiquitinated GABAB receptors and enhanced the expression of total as well as cell surface GABAB receptors. Mutational inactivation of four putative ubiquitination sites in theGABAB1 subunit significantly diminished K63-linked ubiquitination of GABAB receptors and prevented their lysosomal degradation. We identified MIB2 as the E3 ligase triggering K63-linked ubiquitination and lysosomal degradation of GABAB receptors. Finally, we show that sustained activation of glutamate receptors, a condition occurring in brain ischemia that downregulates GABAB receptors, considerably increased the expression of MIB2 and K63-linked ubiquitination of GABAB receptors. Interfering with K63-linked ubiquitination by overexpressing ubiquitin mutants or GABAB1 mutants deficient in K63-linked ubiquitination prevented glutamate-induced down-regulation of the receptors. These findings indicate that K63-linked ubiquitination of GABAB1 at multiple sites by MIB2 controls sorting of GABAB receptors to lysosomes for degradation under physiological and pathological condition.

DOI: https://doi.org/10.1074/jbc.M116.750968

JBC Papers in Press. Published on August 29, 2016 as Manuscript M116.750968
The latest version is at http://www.jbc.org/cgi/doi/10.1074/jbc.M116.750968

K63-Linked Ubiquitination of $GABA_{B1}$ at Multiple Sites by the E3 Ligase Mind Bomb-2 Targets $GABA_B$ Receptors to Lysosomal Degradation

Khaled Zemoura[1], Claudia Trümpler[1] and Dietmar Benke[1,2,3]

From the [1]Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. [2]Neuroscience Center Zurich, University of Zurich and ETH Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland. [3]Drug Discovery Network Zurich (DDNZ), Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

Running title: Lysosomal degradation of $GABA_B$ receptors

To whom the correspondence should be addressed: Dr. Dietmar Benke, Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland, Tel.: 41 44 635 5930, Fax.: 41 44 635 6874, E-mail: benke@pharma.uzh.ch

Keywords: GABA receptor, lysosome, protein degradation, ubiquitination, E3 ubiquitin ligase

Abstract

$GABA_B$ receptors are heterodimeric G protein-coupled receptors, which control neuronal excitability by mediating prolonged inhibition. The magnitude of $GABA_B$ receptor-mediated inhibition essentially depends on the amount of receptors in the plasma membrane. However, the factors regulating cell surface expression of $GABA_B$ receptors are poorly characterized. Cell surface $GABA_B$ receptors are constitutively internalized and either recycled to the plasma membrane or degraded in lysosomes. The signal that sorts $GABA_B$ receptors to lysosomes is currently unknown. Here we show that Mind bomb-2 (MIB2) mediated K63-linked ubiquitination of the $GABA_{B1}$ subunit at multiple sites is the lysosomal sorting signal for $GABA_B$ receptors. We found that inhibition of lysosomal activity in cultured rat cortical neurons increased the fraction of K63-linked ubiquitinated $GABA_B$ receptors and enhanced the expression of total as well as cell surface $GABA_B$ receptors. Mutational inactivation of four putative ubiquitination sites in the $GABA_{B1}$ subunit significantly diminished K63-linked ubiquitination of $GABA_B$ receptors and prevented their lysosomal degradation. We identified MIB2 as the E3 ligase triggering K63-linked ubiquitination and lysosomal degradation of $GABA_B$ receptors. Finally, we show that sustained activation of glutamate receptors, a condition occurring in brain ischemia that downregulates $GABA_B$ receptors, considerably increased the expression of MIB2 and K63-linked ubiquitination of $GABA_B$ receptors. Interfering with K63-linked ubiquitination by overexpressing ubiquitin mutants or $GABA_{B1}$ mutants deficient in K63-linked ubiquitination prevented glutamate-induced down-regulation of the receptors. These findings indicate that K63-linked ubiquitination of $GABA_{B1}$ at multiple sites by MIB2 controls sorting of $GABA_B$ receptors to lysosomes for degradation under physiological and pathological condition.

The number of neurotransmitter receptors at the cell surface available for signalling in neurons needs to be precisely tuned to a given cellular state and must consequently be dynamically adjusted to altered conditions. One key player regulating their amount as well as their life span is protein degradation. Two major cellular protein degradation systems control the number of

neurotransmitter receptors: lysosomes and proteasomes. Interestingly, both systems rely on ubiquitination as a signal that tags most membrane proteins for degradation. For proteasomal degradation primarily K48-linked polyubiquitination and for lysosomal degradation primarily K63-linked polyubiquitination is required (1). Both degradation pathways are involved in the regulation of G protein-coupled $GABA_B$ receptors. $GABA_B$ receptors are heterodimers assembled from $GABA_{B1}$ and $GABA_{B2}$ subunits and are activated by γ-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain, to regulate excitability of neurons. At presynaptic locations, $GABA_B$ receptors suppress neurotransmitter release mainly by inhibiting voltage-gated Ca^{2+} channels whereas at postsynaptic sites they induce slow inhibitory postsynaptic currents by activating Kir3-type K^+ channels (reviewed in (2)). $GABA_B$ receptors are involved in the regulation of all main brain functions ranging from synaptic plasticity (3), neuronal network activity (4,5) to neuronal development (6).

An important factor regulating $GABA_B$ receptor signalling is the dynamic control of their cell surface expression via protein degradation. So far, two mechanisms have been identified: 1) proteasomal degradation of the receptors in the endoplasmic reticulum (ER) and 2) lysosomal degradation of receptors internalized from the plasma membrane. The amount of $GABA_B$ receptors in the endoplasmic reticulum (ER) available for forward trafficking to the cell surface is determined by the rate of their proteasomal degradation via the ERAD (ER associated degradation) machinery (7). Proteasomal degradation of ER residing $GABA_B$ receptors is regulated by the activity state of the neuron via K48-linked ubiquitination of $GABA_{B2}$ and requires interaction of the $GABA_{B2}$ C-terminus with the proteasomal AAA-ATPase Rpt6 (7,8). In contrast, $GABA_B$ receptors at the cell surface are constitutively endocytosed and either recycled to the plasma membrane or degraded in lysosomes (9-13). Lysosomal degradation of $GABA_B$ receptors is most likely mediated via the ESCRT (endosomal sorting complex required for transport) machinery (13), which sorts ubiquitinated membrane proteins to lysosomes (14). Interestingly, the ubiquitin specific protease 14 (USP14) has been implicated in sorting ubiquitinated $GABA_B$ receptors to lysosomal degradation (15). Lysosomal degradation of $GABA_B$ receptors appears to be tightly regulated since excessive activity of glutamate receptors, a condition occurring in brain ischemia, rapidly downregulates $GABA_B$ receptors by preferential sorting them to the lysosomal degradation pathway at the expense of receptor recycling (16-19). The specific signal(s) that sorts $GABA_B$ receptors to lysosomal degradation under normal as well as pathological conditions is currently unknown. Here we show that K63-linked ubiquitination by the E3 ligase mindbomb2 (MIB2) of $GABA_{B1}$ at multiple sites targets $GABA_B$ receptors to the lysosomal degradation pathway.

RESULTS

Lysosomal degradation regulates cell surface expression of $GABA_B$ receptors – $GABA_B$ receptors undergo fast constitutive dynamin and clathrin-dependent endocytosis. Most of the receptors are recycled to the plasma membrane, whereas a minor fraction is sorted to the lysosomal degradation pathway (9-11,20,21). However, it is currently not known whether interfering with lysosomal degradation affects the expression of cell surface expression of $GABA_B$ receptors. Blocking lysosomal degradation in cultured cortical neurons with leupeptin for 12 hours considerably increased total ($GABA_{B1}$, 151 ± 6%; $GABA_{B2}$, 160 ± 7% of control, Fig. 1*A*) as well as cell surface expression of $GABA_B$ receptors ($GABA_{B1}$, 146 ± 9%; $GABA_{B2}$, 147 ± 9% of control Fig. 1*B*) to a similar extent. This suggests that constitutive lysosomal degradation is one factor determining the availability of $GABA_B$ receptors at the cell surface for signaling.

K63-linked ubiquitination is involved in lysosomal degradation of $GABA_B$ receptors – The signal that sort $GABA_B$ receptors to lysosomal degradation is unknown. K48-linked ubiquitination tags proteins for degradation in proteasomes, whereas K63-linked ubiquitination is involved in non-proteolytic functions and can serve as a sorting signal for lysosomal degradation (1). To test whether K63-linked ubiquitination is involved in degrading $GABA_B$ receptors, we transfected neurons with a mutant of ubiquitin that is not able to form K63-linked chains (Ub(K63R)) and analyzed them for cell surface expression of $GABA_B$ receptors. Inhibition of K63-linked ubiquitination by overexpression of Ub(K63R) increased the expression level of cell surface $GABA_B$ receptors ($GABA_{B1}$, 162±12%; $GABA_{B2}$, 136±9% of control neurons transfected with wild type ubiquitin; Fig. 2*A*), suggesting that $GABA_B$ receptor levels are regulated by K63-linked ubiquitination.

Next we tested whether regulation of $GABA_B$ receptor levels by lysosomal degradation requires direct K63-linked ubiquitination of the receptor by *in situ* PLA using antibodies directed against $GABA_{B1}$ and K63-linked ubiquitin. Under basal conditions, $GABA_B$ receptors exhibited K63-linked ubiquitination, which considerably increased upon inhibition of lysosomal activity with leupeptin (164 ± 8 % of control, Fig. 2*B*). In contrast, K48-linked ubiquitination (which targets the receptors to proteasomal degradation (7,8)) remained unaffected by blocking lysosomal activity (Fig. 2*C*). This suggests that direct K63-linked ubiquitination of $GABA_B$ receptors regulates lysosomal degradation of $GABA_B$ receptors.

Identification of K63-linked ubiquitination sites in $GABA_{B1}$ – For identification of K63-linked ubiquitination sites in $GABA_B$ receptors we first determined whether $GABA_{B1}$ or $GABA_{B2}$ is the main target. HEK 293 cells were either transfected with a $GABA_{B1}$ mutant ($GABA_{B1a}$(RSAR)) containing an inactivated ER retention signal, which permits ER exit and cell surface targeting of the subunit when expressed in the absence of $GABA_{B2}$ (22), or with a combination of $GABA_{B1}$ and $GABA_{B2}$ and tested for K63-linked ubiquitination with *in situ* PLA using antibodies directed against $GABA_{B1}$ or $GABA_{B2}$ and K63-linked ubiquitin. We detected no difference in K63-linked ubiquitination between HEK cells expressing $GABA_{B1}$ alone and those expressing $GABA_{B1}$ plus $GABA_{B2}$, suggesting that $GABA_{B1}$ is the main target for K63-linked ubiquitination (Fig. 3*A*).

We then searched for potential lysine residues serving as ubiquitination sites in the $GABA_{B1}$ sequence by an *in silico* analysis. Four lysines with a high probability of being ubiquitinated were identified: two in the cytoplasmatic loop linking transmembrane domains three and four and two in the C terminal domain (Fig. 3*B''*). Inactivation of these sites by mutation to arginine (K->R) yielded the three mutants $GABA_{B1a}$(K697/698R), $GABA_{B1a}$(K892R) and $GABA_{B1a}$(K960R). To test whether these sites are ubiquitinated, HEK293 cells were transfected with either wild type $GABA_{B1a}$ or one of the $GABA_{B1a}$(K->R) mutants along with $GABA_{B2}$ and analyzed for K63-linked ubiquitination by *in situ* PLA. Numerous *in situ* PLA signals in cells transfected with wild type $GABA_{B1a}$ indicated that a fraction of $GABA_{B1}$ is K63-linked ubiquitinated under basal conditions. In contrast, all three mutant $GABA_{B1a}$ displayed strongly reduced K63-linked ubiquitination ($GABA_{B1a}$(K697/698R): 43 ± 3%, $GABA_{B1a}$(K892R): 38 ± 3%, $GABA_{B1a}$(K960R): 37 ±3%, of wild type $GABA_{B1a}$; Fig. 3*B*). This result indicates that lysines 697 and/or 698, lysine 982 and lysine 960 in $GABA_{B1}$ can be K63-linked ubiquitinated under basal conditions.

Ubiquitination of $GABA_{B1}$ regulates cell surface expression of $GABA_B$ receptors – To analyze the effect of K63-linked ubiquitination on cell surface expression of $GABA_B$ receptors, we transfected neurons with wild type $GABA_{B1}$ or $GABA_{B1a}$(K->R) mutants along with $GABA_{B2}$ and immunostained for their total and cell surface expression levels. Total as well as cell surface

expression of all three $GABA_{B1a}$ mutants was considerably increased as compared to transfected wildtype $GABA_{B1a}$ (total, $GABA_{B1a}$(K697/698R): 457 ± 26%, $GABA_{B1a}$(K892R): 511 ± 30%, $GABA_{B1a}$(K960R): 551 ± 22%, of wild type $GABA_{B1}$; cell surface: $GABA_{B1a}$(K697/698R): 508 ± 52%, $GABA_{B1a}$(K892R): 504 ± 48%, $GABA_{B1a}$(K960R): 482.2 ± 42% of wild type $GABA_{B1}$, Fig. 4*A, B*). Likewise, the cell surface expression of $GABA_{B2}$ in neurons transfected with $GABA_{B1a}$(K->R) mutants was significantly increased ($GABA_{B2}$ in $GABA_{B1a}$ (K697/698R) transfected neurons: 158 ± 14%, $GABA_{B2}$ in $GABA_{B1a}$(K892R) transfected neurons: 187 ± 17%, $GABA_{B2}$ in $GABA_{B1a}$(K960R) transfected neurons: 178 ±16% of control; Fig. 4*B*). The considerably lower increase in $GABA_{B2}$ cell surface expression as compared to mutant $GABA_{B1}$ was due to the fact that in case of $GABA_{B1}$ only transfected subunits were assayed (Ha-tagged) while in the case of $GABA_{B2}$ transfected as well as endogenously expressed subunits were detected. The results demonstrate that inactivation of any of the ubiquitination sites in $GABA_{B1}$ (K697/698, K892, K960) decreased or prevented degradation of $GABA_B$ receptors and therefore increased their cell surface expression.

Lysosomal targeting of $GABA_B$ receptors is regulated by ubiquitination of $GABA_{B1}$ – The increased total and cell surface expression levels of $GABA_{B1a}$(K->R) mutants and their reduced K63-linked ubiquitination suggests that ubiquitination of these lysine residues serves as signals for sorting the receptors to lysosomes for degradation. If this is the case, $GABA_{B1a}$(K->R) mutants should be resistant to lysosomal degradation and their expression levels should not increase upon blocking lysosomal degradation. Indeed, in contrast to the expression level of wild type $GABA_{B1}$, those of all three $GABA_{B1a}$(K->R) mutants remained unaffected by inhibition of lysosomal degradation with leupeptin (wild type $GABA_{B1}$: 249 ± 30%, $GABA_{B1a}$ (K697/698R): 111 ± 5%, $GABA_{B1a}$ (K892R): 109 ± 5%, $GABA_{B1a}$ (K960R): 108 ± 4% of control, Fig. 5*A*).

To confirm this finding, we prevented lysosomal degradation by overexpressing a functionally inactive mutant of the small GTPase Rab7 (Rab7(DN)). Rab7 mediates trafficking from early endosomes via late endosomes to lysosomes (23) and therefore overexpression of Rab7(DN) disrupts this pathway. In line with the pharmacological data, overexpression of Rab7(DN) considerably enhanced total expression of wild type $GABA_{B1a}$ but did not significantly affect the expression levels of $GABA_{B1a}$(K->R) mutants (wild type $GABA_{B1a}$: 174 ± 11%, $GABA_{B1a}$ (K697/698R): 105 ± 8%, $GABA_{B1a}$ (K892R): 117 ± 8%, $GABA_{B1a}$ (K960R): 126 ± 9% of control; Fig. 5*B*). This indicates that preventing ubiquitination of specific sites in $GABA_{B1}$ excluded the mutant receptors from entering the endosomal pathway that directs proteins to the lysosome. Therefore, our observations suggest that ubiquitination of multiple lysine residues in $GABA_{B1}$ receptors regulates lysosomal degradation of $GABA_B$ receptors.

The E3 ligase mindbomb-2 (MIB2) mediates K63-linked ubiquitination of $GABA_{B1}$ – In a next step we aimed at identifying the E3 ligase mediating K63-linked ubiquitination of $GABA_B$ receptors. A recent comprehensive proteomic study determined proteins that robustly interact with $GABA_B$ receptors and most likely build basic $GABA_B$ receptor signaling complexes (24). A few E3 ligases, which did not pass their stringent criteria for a robustly associated protein and thus were not regarded as a permanent member of a basic $GABA_B$ receptor signaling complex, emerged in their screens (MIB2, TRIM9 and MYCBP2; additional tested E3 ligases: RNF112, RNF144, RNF167 and RNF152). Upon overexpression of those E3 ligases in neurons we found that the E3 ligase mindbomb-2 (MIB2) significantly reduced cell surface (59 ± 3% of control, Fig. 6*A*) as well as total (78 ± 3% of control, Fig. 6*B*) $GABA_B$ receptor expression. MIB2 extensively colocalized with $GABA_B$ receptors in neurons (Fig. 6*C*) and interacted with

$GABA_B$ receptors as tested by *in situ* PLA (Fig. 6*D*).

Next we analyzed whether MIB2 mediates K63-linked ubiquitination of $GABA_B$ receptors. To demonstrate directly K63-linked ubiquitination of $GABA_B$ receptors by MIB2, we overexpressed MIB2 in neurons and tested for increased K63-linked ubiquitination using *in situ* PLA. In fact, overexpression of MIB2 in neurons increased K63-linked ubiquitination of $GABA_B$ receptors to 156 ± 16% of controls (Fig. 7*A*). This result was corroborated by the observation that overexpression of mutant ubiquitin, which cannot form K63 linkages (Ub(K63R)), inhibited the MIB2 effect on $GABA_B$ receptors (Fig. 7*B*). In contrast overexpression of MIB2 in neurons with either wild type ubiquitin (WT Ub, (59 ± 8% of control), mutant ubiquitin that can only form K63 linkages (Ub(K63), 64 ± 6% of control) or mutant ubiquitin that is deficient in forming K48-inkages (Ub(K48R), 57 ± 6% of control) did not affect MIB2-mediated downregulation of $GABA_B$ receptors (Fig. 7*B*).

To further substantiate that K63-linked ubiquitination is mediated via MIB2 we analyzed the effect of overexpression of MIB2 on the three $GABA_{B1a}$(K->R) mutants, which are partially resistant to K63-linked ubiquitination. In this set of experiments, overexpression of MIB2 reduced cell surface expression of wild type $GABA_{B1}$ to 33 ± 4% of controls (Fig. 8). However, cell surface expression of all three mutants remained unaffected by overexpression of MIB2 (Fig. 8).

Sustained activation of glutamate receptors increases K63-linked ubiquitination of $GABA_B$ receptors via MIB2 – Prolonged activation of glutamate receptors (AMPA as well as NMDA receptors) leads to downregulation of $GABA_B$ receptors via lysosomal degradation (16-18). To investigate whether K63-linked ubiquitination of $GABA_B$ receptors serves as a lysosomal sorting signal in this process, we first tested whether the three $GABA_{B1a}$(K->R) mutants, which are partially resistant to K63-linked ubiquitination, are resistant to glutamate-induced downregulation. In contrast to the cell surface expression of wild type $GABA_{B1}$ (56 ± 8% of control, Fig. 9), the levels of all three $GABA_{B1a}$(K->R) mutants remained unaffected by glutamate ($GABA_{B1a}$(K697/698R): 90 ± 12%, $GABA_{B1a}$(K892R): 115 ± 11%, $GABA_{B1a}$ (K960R): 108 ± 5% of control, Fig. 9). This suggests that K63-linked ubiquitination of $GABA_{B1}$ is the signal for downregulating the receptors.

To directly test for ubiquitination of the receptors in this mechanism, we exposed cortical neurons for 30 min to glutamate and determined K63-linked ubiquitination of the receptors via *in situ* PLA. As expected, sustained activation of glutamate receptors strongly increased K63-linked ubiquitination of $GABA_B$ receptors (203 ± 34% of control, Fig. 10*A*).

Next we tested whether preventing K63-linked ubiquitination inhibits the downregulation of $GABA_B$ receptors after treating neurons with glutamate. For this, cortical neurons were transfected either with wild-type ubiquitin (Ub), a mutant of ubiquitin in which all lysines were mutated to arginines thereby preventing chain elongation and thus any kind of polyubiquitination (Ub(KO)) or with a mutant in which all lysines were mutated to arginines except for K63 (Ub(K63), able to form only K63-linked ubiquitination) and stained for cell surface $GABA_B$ receptors after sustained glutamate application. Glutamate induced downregulation of $GABA_B$ receptors from the plasma membrane in neurons expressing wild type ubiquitin (Ub(WT), 53 ± 5% of control, Fig. 10*B*) or the mutant that only permits K63-linked ubiquitination (Ub(K63), 61 ± 6% of control, Fig. 10*B*) but not in neurons expressing the mutant unable to build polyubiquitin chains (Ub(KO), 95 ± 11% of control, Fig. 10*B*).

Finally, we analyzed whether MIB2 is involved in glutamate-induced downregulation of the receptors. Interestingly, treatment of neurons with glutamate significantly increased MIB2 expression in neurons (15 min glutamate: 150 ± 9% of control, 30 min glutamate: 179 ± 8% of control;

Fig. 11*A*) and strongly increased the interaction of MIB2 with $GABA_B$ receptors as tested with *in situ* PLA (15 min glutamate: 155 ± 13% of control, 30 min glutamate: 218 ± 25% of control; Fig. 11*B*).

These findings suggest that sustained activation of glutamate receptors induces MIB2-mediated K63-linked ubiquitination of $GABA_B$ receptors, promoting their lysosomal degradation.

DISCUSSION

The signaling strength of G protein coupled receptors largely depends on the number of receptors present in the plasma membrane. The mechanisms determining cell surface expression of the receptors include exocytosis, endocytosis, recycling and degradation. $GABA_B$ receptors assemble into heterodimeric $GABA_{B1,2}$ complexes in the ER, which is a prerequisite for their ER exit and forward trafficking to the plasma membrane. After reaching the cell surface, $GABA_B$ receptors are constitutively internalized and either recycled to the plasma membrane or degraded in lysosomes (for a review see (25)). Both, forward trafficking of $GABA_B$ receptors to the cell surface as well as their residence time at the cell surface are tightly regulated by controlled degradation of the receptors. The amount of $GABA_B$ receptors available for forward trafficking to the plasma membrane in the ER is adjusted by proteasomal degradation of the receptors via the ERAD machinery depending on the activity level of the neuron (7,8). On the other hand, the amount of receptors degraded in lysosomes after internalization from the cell surface depends on mechanisms sorting the endocytozed receptors to either lysosomes or recycling endosomes. Interfering with recycling rapidly depletes the receptors from the cell surface by redirecting them to the lysosomal degradation pathway (10). Rapid downregulation of cell surface $GABA_B$ receptors by rerouting the receptors to lysosomes appears to be associated with pathological conditions as it is induced by sustained activation of glutamate receptors, which is a characteristic of brain ischemia (16-19). The factors triggering lysosomal degradation of $GABA_B$ receptors were however unknown. The results of the present study provide evidence that MIB2-mediated K63-linked ubiquitination of $GABA_{B1}$ sorts $GABA_B$ receptors to lysosomes for degradation under physiological and pathological conditions.

We found that pharmacological inhibition of lysosomal activity increased not only total $GABA_B$ receptor levels, which was expected due to the intracellular accumulation of the receptors (9), but also considerably enhanced cell surface expression of the receptors. This finding implies that regulating lysosomal degradation of $GABA_B$ receptors directly affects their cell surface expression, which in turn determines the strength of $GABA_B$ receptors signaling (7). Here we provide evidence that K63-linked ubiquitination is required for lysosomal degradation of $GABA_B$ receptors. First, blocking global K63-linked ubiquitination by overexpressing an ubiquitin mutant (K63R) that is unable to form K63-linked chains significantly increased cell surface expression of $GABA_B$ receptors. Second, blocking lysosomal activity considerably increased the level of K63-linked ubiquitination of $GABA_B$ receptors while leaving the level of K48-linked ubiquitination, which tags the receptors for proteasomal degradation (7), unaffected. Mutational inactivation of potential ubiquitination sites in $GABA_{B1}$ (K697/698, K892 and K960) strongly decreased K63-linked ubiquitination of $GABA_B$ receptors containing the respective $GABA_{B1}$ mutant and prevented their lysosomal degradation as indicated by their dramatically increased expression level and insensitivity to the effect of blocking lysosomal degradation (either by inhibiting lysosomal proteases by leupeptin or by overexpression of a functionally inactive mutant of Rab 7, which inhibits transport of cargo from late endosomes to the lysosome and blocks lysosome biogenesis). Any of the three $GABA_{B1}$ mutants (K697/698R, K892R and K960R) appeared to completely prevent lysosomal degradation of the receptors, suggesting that ubiquitination of K697/698, K892 and K960 in

$GABA_{B1}$ is mandatory for lysosomal degradation of $GABA_B$ receptors. A similar situation was reported for targeting EGF receptors to lysosomal degradation. Multiple 63K-linked ubiquitination sites were identified and mutation of each site prevented degradation of the receptors (26). It is currently unclear at which stage of intracellular sorting K697/698, K892 and K960 in $GABA_{B1}$ need to be ubiquitinated. They may be ubiquitinated simultaneously at a certain sorting step or, alternatively, they may be sequentially ubiquitinated at distinct sorting checkpoints. Addressing this issue in relation to the ESCRT pathway for sorting the receptors to lysosomes is an important question that requires further investigation.

Lysosomal degradation of G protein coupled receptors is predominantly mediated via the ESCRT machinery (27), which guides mono- and K63-linked ubiquitinated membrane proteins to lysosomes (28). Therefore, our observation that K63-linked ubiquitination tags $GABA_B$ receptors for lysosomal degradation indicates that the ESCRT machinery also sorts $GABA_B$ receptors to lysosomes. This view is supported by the finding that the ESCRT I complex component TGS101 (29) is required for lysosomal degradation of $GABA_B$ receptors (13). In addition, the deubiquitination enzymes USP14 has been implicated in lysosomal degradation of $GABA_B$ receptors (15). Deubiquitinaton of proteins is an integral part of ESCRT-mediated degradation. Deubiquitinases associated with the ESCRT-0 complex are thought to rescue proteins from degradation by deubiquitination at an early step of lysosomal targeting, whereas deubiquitinases recruited to ESCRT-III recycle ubiquitin before the cargo protein is being degraded in the lysosome (14). However, USP14 appears not to be involved in these classical functions. Instead, USP14 interacts with $GABA_B$ receptors and contributes to their lysosomal targeting independent of its deubiquitinating activity, in an as yet undefined way (15).

So far, information on the E3 ubiquitin ligases mediating ubiquitination of $GABA_B$ receptors is almost entirely lacking. We previously found that the prototypical ERAD E3 ligase Hrd1 interacts with $GABA_B$ receptors residing in the ER and is most likely responsible for K48-linked ubiquitination of $GABA_{B2}$, which tags the receptors for proteasomal degradation (7). Here we identified MIB2 as the E3 ubiquitin ligase mediating K63-linked ubiquitination of $GABA_{B1}$, tagging the receptors for lysosomal degradation. MIB2 was detected in a proteomic screen, but did not fulfill the rigorous criteria of the authors for a robustly $GABA_B$ receptor associated protein (24). However, we found that MIB2 in fact colocalized with $GABA_B$ receptors in neurons and interacts with $GABA_B$ receptor complexes as tested by *in situ* PLA.

MIB2 belongs to the class of RING (really interesting new gene) domain E3 ligases composed of two separate substrate recognition domains in its N terminal portion and two RING domains with the ubiquitin ligase activity in the C terminal portion (30). The best described function of MIB2 is the ubiquitination and internalization of Notch ligands (31). Because Notch signaling in the adult brain is involved in synaptic plasticity, memory and learning, MIB2-deficient mice displayed impaired hippocampal long-term potentiation (LTP) and spatial memory as well as contextual fear memory (32). Apart from regulating Notch signaling, MIB2 has been shown to control diverse systems. For instance, it mediates K63-linked ubiquitination of TANK-binding kinase 1 resulting in interferon regulatory factor 3/7 activation (33), it controls NF-κB activation (34) and it ubiquitinates the NR2B subunit of NMDA receptors to downregulate their activity (35). Our experiments using mutant ubiquitin, $GABA_B$ receptor ubiquitination deficient mutants as well as *in situ* PLA indicate that MIB2 mediates K63-linked ubiquitination of $GABA_{B1}$ and thereby controls their lysosomal degradation.

To verify the importance of MIB2-mediated K63-linked ubiquitination for lysosomal degradation, we tested its involvement in an experimental setting that mimics an important aspect of cerebral ischemia (sustained activation of glutamate receptors), which leads to a rapid downregulation of $GABA_B$ receptors via lysosomal degradation (16-19). Interestingly, prolonged activation of neurons with glutamate considerably increased the expression levels of MIB2 within 15-30 minutes. This rapid upregulation of MIB2 might be enabled by the autoubiquitination activity of MIB2. The turnover of MIB2 has been suggested to be regulated by the interplay of its autoubiquitinating activity, leading to its proteasomal degradation, and the activity of interacting deubiquitinating enzymes (36). Thus, it is conceivable that sustained activation of glutamate receptors may increase the activity of an MIB2-associated deubiquitinase, which prevents autoubiquitination and proteasomal degradation of MIB2. The enhanced expression of MIB2 was accompanied with an increased interaction of MIB2 with $GABA_B$ receptors and an elevated K63-linked ubiquitination. Interfering with K63-linked ubiquitination by overexpressing ubiquitin mutants or our $GABA_{B1a}$(K->R) mutants prevented glutamate-induced downregulation of the receptors. These results indicate that MIB2-mediated K63-linked ubiquitination is indispensable for downregulating the receptors via the lysosomal pathway and that the level of lysosomal degradation of the receptors is, at least in part, dependent on the expression level of MIB2.

In conclusion, our data suggest that MIB2 mediated K63-linked ubiquitination of $GABA_{B1}$ sorts $GABA_B$ receptors to lysosomes for degradation under physiological as well as pathological conditions.

EXPERIMENTAL PROCEDURES

Antibodies – The following antibodies were used: mouse anti-HA (1:1000 for immuno-fluorescence, 1:500 for *in situ* PLA, Sigma-Aldrich), rabbit $GABA_{B1b}$ directed against the N-terminus of $GABA_{B1b}$ (affinity-purified, 1:200 for immunofluorescence, custom made by GenScript) (37), rabbit $GABA_{B2}$ directed against the N-terminus of $GABA_{B2}$ (affinity-purified, 1:500 for immunofluorescence; custom made by GenScript) (38), guinea pig $GABA_{B2}$ (1:500 for immunofluorescence; Millipore Cat. # AB2255, Lot # 2484228), mouse $GABA_{B1}$ (1:100 for PLA; NeuroMab, clone N93A/49, Cat. # 75-183), rabbit ubiquitin K48-specific (clone Apu2, 1:50 for *in situ* PLA; Millipore, Cat. # 05-1307, Lot # 2385989), rabbit ubiquitin K63-specific (clone Apu3, 1:50 for *in situ* PLA; Millipore, Cat. # 05-1308, Lot # 2575910), rabbit MIB2 (1: 1000 for immunofluorescence, 1:250 for PLA; MyBioSource Cat. # MBS2014413, Lot # A20160407515). Secondary antibodies were purchased from Jackson ImmunoResearch Laboratories labeled with either Alexa Fluor 488 (1:800), Cy-3 (1:500) or Cy-5 (1:300).

Drugs - The following chemicals were used for this study: glutamate (50 µM, Sigma-Aldrich), leupeptin (100 µM, Sigma-Aldrich).

Plasmids – The following DNAs were used: HA-tagged $GABA_{B1a}$ (39), $GABA_{B1a}$(RSAR) (22), $GABA_{B2}$ (40), HA-tagged ubiquitin (Addgene plasmid 17608), HA-tagged ubiquitin (KO) (Addgene plasmid 17603), HA-tagged ubiquitin K63 (Addgene plasmid 17606) and HA-tagged ubiquitin K48R (Addgene plasmid 17604) (41); HA-tagged ubiquitin K63R was kindly provided by L.-Y. Liu-Chen, Temple University, Philadelphia, USA; wild type EGFP-tagged rab7 (Addgene plasmid 12605) and the functionally inactive mutant EGFP-tagged rab7(DN) (Addgene plasmid 12660) (42); HA-tagged MIB2 (Addgene plasmid 33312) (34).

Mutation of $GABA_{B1}$ - Lysines 697, 698, 892 and 960 in $GABA_{B1a}$ were mutated to arginines using the QuikChange II XL site-directed mutagenesis kit from Stratagene according to the manufacturer's instructions.

Culture and transfection of cortical neurons – Primary neuronal cultures of cerebral cortex were

prepared from 18 day old embryos of Wistar rats as described previously (10). Neurons were used after 11 to 15 days in culture. Plasmid DNA was transfected into neurons by magnetofection using Lipofectamine 2000 (Invitrogen) and CombiMag (OZ Biosciences) as detailed in (43).

Immunocytochemistry and confocal laser scanning microscopy – Immunofluorescence staining was performed as described previously (10,44). For selective detection of cell surface $GABA_B$ receptors living neurons were incubated with antibodies recognizing the extracellular located N-terminal domain of $GABA_{B1}$ or $GABA_{B2}$ for one hour at 4°C. For analysis of total $GABA_B$ receptors, neurons were fixed with 4% paraformaldehyde for 15-20 min at room temperature and permeabilized with 0.2% Triton X-100 before immunostaining.

Stained neurons were analyzed by laser scanning confocal microscopy (LSM 510 Meta or LSM 700, Zeiss). Images of eight optical sections spaced by 0.3 µm were recorded with a 100x plan-fluar oil differential interference contrast objective (1.45 NA, Zeiss) at a resolution of 1024 x 1024 pixels. Quantitative analysis of total and cell surface staining was performed as described in (44).

In-cell Western assay – Total $GABA_B$ receptor expression of neurons cultured in 96-well plates was analyzed using the in-cell Western assay exactly as described previously (17). Fluorescence signals generated by $GABA_{B1}$ and $GABA_{B2}$ antibodies were normalized to actin signals determined simultaneously in the same cultures.

In situ proximity ligation assay (in situ PLA) – *In situ* PLA is an antibody based technology for the detection of protein-protein interactions and posttranslational modifications of proteins in cells *in situ* (45,46). The *in situ* PLA was performed using Duolink PLA probes and detection reagents (Olink Bioscience, Sigma-Aldrich) according to the manufacturer's instructions as described previously (44). Here we applied *in situ* PLA primarily for the detection and quantification of $GABA_B$ receptor ubiquitination using mouse $GABA_{B1}$ or mouse HA antibodies together with rabbit antibodies specifically detecting K48-linked or K63-linked ubiquitin. Quantification was done by counting individual *in situ* PLA spots using the Mac Biophotonics Image J software. The number of spots was normalized to the area analyzed and to the expression level of $GABA_B$ receptors.

Statistics – The statistical analyses were done with GraphPad Prism 5. The tests used and p values are given in the figure legends. Differences were considered statistically significant when $p < 0.05$.

Acknowledgments: This study was supported by the Swiss National Science Foundation (grant 31003A_138382 to D.B.). We are grateful to Dr. Jean-Marc Fritschy for his continued support in microscopy and for providing E18 rat cortex and to Giovanna Bosshard for dissecting the E18 rat cortex. We thank Marisa Schenkel for introducing the mutations in $GABA_{B1}$ and Thomas Grampp for excellent technical assistance.

Conflict of interest: The authors declare that they have no conflicts of interest with the content of this article.

Author contribution: KZ conceived and conducted most of the experiments, analyzed data and contributed to writing the manuscript. Claudia Trümpler conducted and analyzed the experiments shown in figures 3*B*, 4 and 5. DB conceived the project, analyzed data and wrote the manuscript.

REFERENCES

1. Komander, D., and Rape, M. (2012) The ubiquitin code. *Annu. Rev. Biochem.* **81**, 203-229
2. Benke, D., Zemoura, K., and Maier, P. J. (2012) Modulation of cell surface $GABA_B$ receptors by desensitization, trafficking and regulated degradation. *World J. Biol. Chem.* **3**, 61-72
3. Pinard, A., Seddik, R., and Bettler, B. (2010) $GABA_B$ receptors: physiological functions and mechanisms of diversity. *Adv. Pharmacol.* **58**, 231-255
4. Craig, M. T., and McBain, C. J. (2014) The emerging role of $GABA_B$ receptors as regulators of network dynamics: fast actions from a 'slow' receptor? *Curr. Opin. Neurobiol.* **26**, 15-21
5. Kohl, M. M., and Paulsen, O. (2010) The roles of $GABA_B$ receptors in cortical network activity. *Adv. Pharmacol.* **58**, 205-229
6. Gaiarsa, J. L., and Porcher, C. (2013) Emerging neurotrophic role of $GABA_B$ receptors in neuronal circuit development. *Front. Cell. Neurosci.* **7**, 206
7. Zemoura, K., Schenkel, M., Acuna, M. A., Yevenes, G. E., Zeilhofer, H. U., and Benke, D. (2013) Endoplasmic reticulum-associated degradation (ERAD) controls cell surface expression of γ-aminobutyric acid, type B receptors. *J. Biol. Chem.* **288**, 34897-34905
8. Zemoura, K., and Benke, D. (2014) Proteasomal degradation of γ-aminobutyric $acid_B$ receptors is mediated by the interaction of the $GABA_{B2}$ C terminus with the proteasomal ATPase Rtp6 and regulated by neuronal activity. *J. Biol. Chem.* **289**, 7738-7746
9. Grampp, T., Sauter, K., Markovic, B., and Benke, D. (2007) γ-Aminobutyric acid type B receptors are constitutively internalized via the clathrin-dependent pathway and targeted to lysosomes for degradation. *J. Biol. Chem.* **282**, 24157-24165
10. Grampp, T., Notz, V., Broll, I., Fischer, N., and Benke, D. (2008) Constitutive, agonist-accelerated, recycling and lysosomal degradation of $GABA_B$ receptors in cortical neurons. *Mol. Cell. Neurosci.* **39**, 628-637
11. Vargas, K. J., Terunuma, M., Tello, J. A., Pangalos, M. N., Moss, S. J., and Couve, A. (2008) The availability of surface $GABA_B$ receptors is independent of γ-aminobutyric acid but controlled by glutamate in central neurons. *J. Biol. Chem.* **283**, 24641-24648
12. Hannan, S., Wilkins, M. E., Dehghani-Tafti, E., Thomas, P., Baddeley, S. M., and Smart, T. G. (2011) $GABA_B$ receptor internalisation is regulated by the R2 subunit. *J. Biol. Chem.* **286**, 24324-24335
13. Kantamneni, S., Holman, D., Wilkinson, K. A., Correa, S. A., Feligioni, M., Ogden, S., Fraser, W., Nishimune, A., and Henley, J. M. (2008) GISP binding to TSG101 increases $GABA_B$ receptor stability by down-regulating ESCRT-mediated lysosomal degradation. *J. Neurochem.* **107**, 86-95
14. Raiborg, C., and Stenmark, H. (2009) The ESCRT machinery in endosomal sorting of ubiquitylated membrane proteins. *Nature* **458**, 445-452
15. Lahaie, N., Kralikova, M., Prezeau, L., Blahos, J., and Bouvier, M. (2016) Post-endocytotic deubiquitination and degradation of the metabotropic γ-aminobutyric acid receptor by the ubiquitin-specific protease 14. *J. Biol. Chem.* **291**, 7156-7170
16. Guetg, N., Aziz, S. A., Holbro, N., Turecek, R., Rose, T., Seddik, R., Gassmann, M., Moes, S., Jenoe, P., Oertner, T. G., Casanova, E., and Bettler, B. (2010) NMDA receptor-dependent $GABA_B$ receptor internalization via CaMKII phosphorylation of serine 867 in $GABA_{B1}$. *Proc. Natl. Acad. Sci. U S A* **107**, 13924-13929
17. Maier, P. J., Marin, I., Grampp, T., Sommer, A., and Benke, D. (2010) Sustained glutamate receptor activation down-regulates $GABA_B$ receptors by shifting the balance from recycling to lysosomal degradation. *J. Biol. Chem.* **285**, 35606-35614
18. Terunuma, M., Vargas, K. J., Wilkins, M. E., Ramirez, O. A., Jaureguiberry-Bravo, M., Pangalos, M. N., Smart, T. G., Moss, S. J., and Couve, A. (2010) Prolonged activation of NMDA receptors promotes dephosphorylation and alters postendocytic sorting of $GABA_B$ receptors. *Proc. Natl. Acad. Sci. U S A* **107**, 13918-13923

19. Kantamneni, S., Gonzalez-Gonzalez, I. M., Luo, J., Cimarosti, H., Jacobs, S. C., Jaafari, N., and Henley, J. M. (2014) Differential regulation of $GABA_B$ receptor trafficking by different modes of N-methyl-D-aspartate (NMDA) receptor signaling. *J. Biol. Chem.* **289**, 6681-6694
20. Laffray, S., Tan, K., Dulluc, J., Bouali-Benazzouz, R., Calver, A. R., Nagy, F., and Landry, M. (2007) Dissociation and trafficking of rat $GABA_B$ receptor heterodimer upon chronic capsaicin stimulation. *Eur. J. Neurosci.* **25**, 1402-1416
21. Wilkins, M. E., Li, X., and Smart, T. G. (2008) Tracking cell surface $GABA_B$ receptors using an α-bungarotoxin tag. *J. Biol. Chem.* **283**, 34745-34752
22. Pagano, A., Rovelli, G., Mosbacher, J., Lohmann, T., Duthey, B., Stauffer, D., Ristig, D., Schuler, V., Meigel, I., Lampert, C., Stein, T., Prezeau, L., Blahos, J., Pin, J. P., Froestl, W., Kuhn, R., Heid, J., Kaupmann, K., and Bettler, B. (2001) C-terminal interaction is essential for surface trafficking but not for heteromeric assembly of $GABA_B$ receptors. *J. Neurosci.* **21**, 1189-1202
23. Ng, E. L., Gan, B. Q., Ng, F., and Tang, B. L. (2012) Rab GTPases regulating receptor trafficking at the late endosome-lysosome membranes. *Cell. Biochem. Funct.* **30**, 515-523
24. Schwenk, J., Perez-Garci, E., Schneider, A., Kollewe, A., Gauthier-Kemper, A., Fritzius, T., Raveh, A., Dinamarca, M. C., Hanuschkin, A., Bildl, W., Klingauf, J., Gassmann, M., Schulte, U., Bettler, B., and Fakler, B. (2015) Modular composition and dynamics of native $GABA_B$ receptors identified by high-resolution proteomics. *Nat. Neurosci.* **19**, 233-242
25. Benke, D. (2010) Mechanisms of $GABA_B$ receptor exocytosis, endocytosis, and degradation. *Adv. Pharmacol.* **58**, 93-111
26. Huang, F., Kirkpatrick, D., Jiang, X., Gygi, S., and Sorkin, A. (2006) Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain. *Mol. Cell* **21**, 737-748
27. Dores, M. R., and Trejo, J. (2014) Atypical regulation of G protein-coupled receptor intracellular trafficking by ubiquitination. *Curr. Opin. Cell Biol.* **27**, 44-50
28. Lauwers, E., Jacob, C., and Andre, B. (2009) K63-linked ubiquitin chains as a specific signal for protein sorting into the multivesicular body pathway. *J. Cell. Biol.* **185**, 493-502
29. Hurley, J. H., and Emr, S. D. (2006) The ESCRT complexes: structure and mechanism of a membrane-trafficking network. *Annu. Rev. Biophys. Biomol. Struct.* **35**, 277-298
30. Guo, B., McMillan, B. J., and Blacklow, S. C. (2016) Structure and function of the Mind bomb E3 ligase in the context of Notch signal transduction. *Curr. Opin. Struct. Biol.* **41**, 38-45
31. Koo, B. K., Yoon, K. J., Yoo, K. W., Lim, H. S., Song, R., So, J. H., Kim, C. H., and Kong, Y. Y. (2005) Mind bomb-2 is an E3 ligase for Notch ligand. *J. Biol. Chem.* **280**, 22335-22342
32. Kim, S., Kim, T., Lee, H. R., Kong, Y. Y., and Kaang, B. K. (2015) Mind bomb-2 regulates hippocampus-dependent memory formation and synaptic plasticity. *Korean J. Physiol. Pharmacol.* **19**, 515-522
33. Ye, J. S., Kim, N., Lee, K. J., Nam, Y. R., Lee, U., and Joo, C. H. (2014) Lysine 63-linked TANK-binding kinase 1 ubiquitination by mindbomb E3 ubiquitin protein ligase 2 is mediated by the mitochondrial antiviral signaling protein. *J. Virol.* **88**, 12765-12776
34. Stempin, C. C., Chi, L., Giraldo-Vela, J. P., High, A. A., Hacker, H., and Redecke, V. (2011) The E3 ubiquitin ligase mind bomb-2 (MIB2) protein controls B-cell CLL/lymphoma 10 (BCL10)-dependent NF-κB activation. *J. Biol. Chem.* **286**, 37147-37157
35. Jurd, R., Thornton, C., Wang, J., Luong, K., Phamluong, K., Kharazia, V., Gibb, S. L., and Ron, D. (2008) Mind bomb-2 is an E3 ligase that ubiquitinates the N-methyl-D-aspartate receptor NR2B subunit in a phosphorylation-dependent manner. *J. Biol. Chem.* **283**, 301-310
36. Tseng, L. C., Zhang, C., Cheng, C. M., Xu, H., Hsu, C. H., and Jiang, Y. J. (2014) New classes of mind bomb-interacting proteins identified from yeast two-hybrid screens. *PLoS One* **9**, e93394
37. Benke, D., Honer, M., Michel, C., Bettler, B., and Mohler, H. (1999) γ-Aminobutyric acid type B receptor splice variant proteins GBR1a and GBR1b are both associated with GBR2 *in situ* and display differential regional and subcellular distribution. *J. Biol. Chem.* **274**, 27323-27330

38. Benke, D., Michel, C., and Mohler, H. (2002) Structure of $GABA_B$ receptors in rat retina. *J. Recept. Signal Transduc.* **22**, 253-266
39. Kaupmann, K., Huggel, K., Heid, J., Flor, P. J., Bischoff, S., Mickel, S. J., McMaster, G., Angst, C., Bittiger, H., Froestl, W., and Bettler, B. (1997) Expression cloning of $GABA_B$ receptors uncovers similarity to metabotropic glutamate receptors. *Nature* **386**, 239-246
40. Kaupmann, K., Malitschek, B., Schuler, V., Heid, J., Froestl, W., Beck, P., Mosbacher, J., Bischoff, S., Kulik, A., Shigemoto, R., Karschin, A., and Bettler, B. (1998) $GABA_B$-receptor subtypes assemble into functional heteromeric complexes. *Nature* **396**, 683-687
41. Lim, K. L., Chew, K. C., Tan, J. M., Wang, C., Chung, K. K., Zhang, Y., Tanaka, Y., Smith, W., Engelender, S., Ross, C. A., Dawson, V. L., and Dawson, T. M. (2005) Parkin mediates nonclassical, proteasomal-independent ubiquitination of synphilin-1: implications for Lewy body formation. *J. Neurosci.* **25**, 2002-2009
42. Choudhury, A., Dominguez, M., Puri, V., Sharma, D. K., Narita, K., Wheatley, C. L., Marks, D. L., and Pagano, R. E. (2002) Rab proteins mediate Golgi transport of caveola-internalized glycosphingolipids and correct lipid trafficking in Niemann-Pick C cells. *J. Clin. Invest.* **109**, 1541-1550
43. Buerli, T., Pellegrino, C., Baer, K., Lardi-Studler, B., Chudotvorova, I., Fritschy, J. M., Medina, I., and Fuhrer, C. (2007) Efficient transfection of DNA or shRNA vectors into neurons using magnetofection. *Nat. Protoc.* **2**, 3090-3101
44. Maier, P. J., Zemoura, K., Acuna, M. A., Yevenes, G. E., Zeilhofer, H. U., and Benke, D. (2014) Ischemia-like oxygen and glucose deprivation mediates down-regulation of cell surface γ-aminobutyric $acid_B$ receptors via the endoplasmic reticulum (ER) stress-induced transcription factor CCAAT/enhancer-binding protein (C/EBP)-homologous protein (CHOP). *J. Biol. Chem.* **289**, 12896-12907
45. Leuchowius, K. J., Jarvius, M., Wickstrom, M., Rickardson, L., Landegren, U., Larsson, R., Soderberg, O., Fryknas, M., and Jarvius, J. (2010) High content screening for inhibitors of protein interactions and post-translational modifications in primary cells by proximity ligation. *Mol. Cell Proteomics* **9**, 178-183
46. Soderberg, O., Gullberg, M., Jarvius, M., Ridderstrale, K., Leuchowius, K. J., Jarvius, J., Wester, K., Hydbring, P., Bahram, F., Larsson, L. G., and Landegren, U. (2006) Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat. Methods* **3**, 995-1000

FIGURE LEGENDS

FIGURE 1. **The expression level of $GABA_B$ receptors is controlled by lysosomes.**
A, The total expression level of $GABA_B$ receptors is increased in neurons after blocking lysosomal activity. Cortical neurons were incubated for 12 hours with 100 µM leupeptin (Leup) followed by immunostaining for total $GABA_{B1}$ and $GABA_{B2}$ as well as for actin using the in-cell Western technique. Neurons not treated with leupeptin served as controls (Ctrl). Right, representative images of an in-cell Western. Left, the graph shows the quantification of fluorescence intensities normalized to the corresponding actin signals. Fluorescence intensities for $GABA_{B1}$ and $GABA_{B2}$ in control neurons were set to 100%. The data represent the mean ± SEM of 30 cultures from three independent experiments. *** $p< 0.0001$; two-tailed unpaired t-test.
B, Expression of cell surface $GABA_B$ receptors is increased in neurons after inhibiting lysosomal activity. Cortical neurons were treated as indicated in *A* and immunostained for cell surface $GABA_{B1}$ and $GABA_{B2}$. Left, representative images of the soma of stained neurons. Scale bar, 5 µm. Right, the graphs show the quantification of fluorescence intensities. Fluorescence intensities for $GABA_{B1}$ and $GABA_{B2}$ in control neurons were set to 100%. The data represent the mean ± SEM of 30-40 neurons from three independent experiments. *** $p< 0.0001$; two-tailed unpaired t-test.

FIGURE 2. **The expression level of $GABA_B$ receptors is regulated by K63-linked ubiquitination.**
A, Interference with K63-linked ubiquitination increased the expression level of cell surface $GABA_B$ receptors. Neurons were transfected with wild type ubiquitin (Ub) or a ubiquitin mutant unable to form K63-linked chains (Ub(K63R)) and analyzed for $GABA_B$ receptor expression using $GABA_{B1}$ as well as $GABA_{B2}$ antibodies. Left, representative images of stained neuronal somata (scale bar, 5 µm). Right, quantification of fluorescence intensities. The fluorescence signal of neurons transfected with wild type ubiquitin was set to 100%. The data represent the mean ± SEM of 30-34 neurons from three ($GABA_{B1}$) and two ($GABA_{B2}$) independent experiments. ** $p< 0.004$, *** $p< 0.0001$; two-tailed unpaired t-test.
B, Inhibition of lysosomal activity enhanced K63-linked ubiquitination of $GABA_B$ receptors. Cortical neurons were incubated for 12 hour with or without (control) 100 µM leupeptin and analyzed for K63-linked ubiquitination by *in situ PLA* using antibodies directed against $GABA_{B1}$ and K63-linked ubiquitin (white dots in representative images, scale bar: 5 µm). Right, quantification of *in situ* PLA signals. The data represent the mean ± SEM of 30-40 neurons from three independent experiments. *** $p< 0.00001$; two-tailed unpaired t-test.
C, Inhibition of lysosomal activity did not affect K48-linked ubiquitination of $GABA_B$ receptors. Cortical neurons were treated as in *B* and analyzed for K48-linked ubiquitination by *in situ PLA* using antibodies directed against $GABA_{B1}$ and K48-linked ubiquitin (white dots in representative images, scale bar: 5 µm). Right, quantification of *in situ* PLA signals. The data represent the mean ± SEM of 27-37 neurons from three independent experiments; n.s., $p> 0.05$; two-tailed unpaired t-test.

FIGURE 3. **Identification of K63-linked ubiquitination sites in $GABA_{B1}$.**
A, $GABA_{B1}$ is the main target for K63-linked ubiquitination. HEK 293 cells were either transfected with a $GABA_{B1}$ mutant containing an inactivated ER retention signal ($GABA_{B1a}$(RSAR)), which permits ER exit and cell surface targeting of the subunit when expressed alone, or with $GABA_{B1}$ and $GABA_{B2}$ and tested for K63-linked ubiquitination by *in situ* PLA using $GABA_{B1}$ antibodies in combination with an antibody detecting K63-linked ubiquitin (white dots in representative images, scale bar: 7 µm). The data represent

the mean ± SEM of 47-49 neurons from three independent experiments. ns, p> 0.05; two-tailed unpaired t-test.

B, Decreased K63-linked ubiquitination of $GABA_{B1}$(K->R) mutants. Cortical neurons were transfected with HA-tagged wild type $GABA_{B1a}$, HA-tagged $GABA_{B1a}$(K697/698R), HA-tagged $GABA_{B1a}$(K892R) or HA-tagged $GABA_{B1a}$(K960R) together with wild type $GABA_{B2}$ and analyzed for K63-linked ubiquitination by *in situ* PLA using antibodies directed against the HA-tag and K63-linked ubiquitin (white dots in representative images, scale bar: 7 µm). B', quantification of *in situ* PLA signals. B'', Cartoon depicting the location of K->R mutations in $GABA_{B1}$. The data represent the mean ± SEM of 26-35 neurons from three independent experiments. ns, p> 0.05; *** p< 0.0001; one way ANOVA, Bonferoni's Multiple Comparison test.

FIGURE 4. **$GABA_{B1}$(K->R) mutants exhibit increased total and cell surface expression.**

A, Increased total expression levels of $GABA_{B1a}$(K->R) mutants. Neurons were transfected with HA-tagged wild type $GABA_{B1a}$, HA-tagged $GABA_{B1a}$(K697/698R), HA-tagged $GABA_{B1a}$(K892R) or HA-tagged $GABA_{B1a}$(K960R) together with wild type $GABA_{B2}$ and analyzed for the expression level of transfected $GABA_{B1}$ using antibodies directed against the HA-tag. Left, representative images (scale bar: 7 µm). Right, quantification of fluorescence signals. The fluorescence signals of neurons transfected with wild type $GABA_{B1}$ was set to 100%. The data represent the mean ± SEM of 23-27 neurons per experimental condition derived from three independent experiments. *** p< 0.0001; one way ANOVA, Dunnett's Multiple Comparison test.

B, Increased cell surface expression levels of $GABA_{B1a}$(K->R) mutants. Neurons were transfected with HA-tagged wild type $GABA_{B1a}$, HA-tagged $GABA_{B1a}$(K697/698R), HA-tagged $GABA_{B1a}$(K892R) or HA-tagged $GABA_{B1a}$(K960R) together with wild type $GABA_{B2}$ and analyzed for cell surface expression levels of transfected $GABA_{B1}$ as well as transfected plus endogenous $GABA_{B2}$ using antibodies directed against the HA-tag and $GABA_{B2}$, respectively. Left, representative images (scale bar: 7 µm). Right, quantification of fluorescence signals. The fluorescence signals of neurons transfected with wild type $GABA_{B1}$ or wild type $GABA_{B2}$, respectively, was set to 100%. The data represent the mean ± SEM of 26-28 neurons per experimental condition derived from three independent experiments. ** p< 0.001; *** p< 0.0001; one way ANOVA, Dunnett's Multiple Comparison test.

FIGURE 5. **The expression levels of $GABA_{B1a}$(K->R) mutants are unaffected by inhibition of lysosomal degradation**

A, Total expression levels of $GABA_{B1a}$(K->R) mutants are unaffected by blocking lysosomal activity with leupeptin. Neurons were transfected with HA-tagged wild type $GABA_{B1a}$ or HA-tagged $GABA_{B1a}$(K->R) mutants together with $GABA_{B2}$, incubated with 100 µM leupeptin for 12 hours followed by immunostaining for transfected HA-tagged $GABA_{B1}$ using HA-antibodies. Left, representative images of untreated neurons (control, left) and of neurons incubated with leupeptin (right, scale bar: 7 µm). Right, quantification of fluorescence intensities. The fluorescence intensity of $GABA_{B1a}$ from untreated neurons (control) was set to 100%. The data represent the mean ± SEM of 27-34 neurons per experimental condition derived from three independent experiments. *** p< 0.0001, two-tailed unpaired t-test.

B, Total expression levels of $GABA_{B1a}$(K->R) mutants are unaffected upon blocking lysosomal targeting by inactivation of Rab7. Neurons were transfected with HA-tagged wild type $GABA_{B1a}$ or $GABA_{B1a}$(K->R) mutants together with $GABA_{B2}$ and with either wild type Rab7 or with a non-functional mutant of Rab7 (Rab7(DN)) and analyzed for total expression levels of transfected $GABA_{B1}$ using HA antibodies. Left, representative images depicting total expression of transfected $GABA_{B1a}$ (scale bar: 7 µm). Right,

quantification of fluorescence intensities. The fluorescence intensities of $GABA_{B1a}$ co-expressed with wild type Rab7 were set to 100%. The data represent the mean ± SEM of 27-34 neurons derived from three independent experiments. *** p< 0.0001, two-tailed unpaired t-test.

FIGURE 6. **The E3 ligase MIB2 colocalizes with $GABA_B$ receptors and affects their expression level.**
A, *B*, Overexpression of MIB2 in neurons reduced expression levels of $GABA_B$ receptors. Neurons were either transfected with EGFP (controls) or MIB2 and analyzed for cell surface (*A*) or total (*B*) expression of $GABA_B$ receptors. Left, representative images (scale bar: 5 µm). Right, quantification of fluorescence intensities. The data represent the mean ± SEM of 30 (*A*) and 45 (*B*) neurons derived from two independent experiments. *** p< 0.0001, two-tailed unpaired t-test.
C, MIB2 and $GABA_B$ receptors extensively colocalize in cortical neurons. Neurons were simultaneously stained for $GABA_{B1}$ (red) and MIB2 (green). Scale bar: 5 µm.
D) $GABA_B$ receptors interact with MIB2. Neurons were stained for $GABA_{B1}$ and MIB2 and analyzed for interaction via *in situ* PLA. Scale bar: 5 µm.

FIGURE 7. **MIB2-induced downregulation of $GABA_B$ receptors is mediated by K63-linked ubiquitination.**
A, MIB2 mediates K63-linked ubiquitination of $GABA_B$ receptors. Neurons were transfected with EGFP (control) or MIB2 and tested for K63-linked ubiquitination of $GABA_B$ receptors using *in situ* PLA using antibodies directed against $GABA_{B1}$ and K63-linked ubiquitin (white dots in representative images, scale bar: 5 µm). The graph depicts quantification of the *in situ* PLA signals. The data represent the mean ± SEM of 20 neurons per condition derived from two independent experiments. * p< 0.05, two-tailed unpaired t-test.
B, MIB2-induced downregulation of $GABA_B$ receptors is mediated by K63-linked ubiquitination. Neurons were transfected with either wild type ubiquitin (WT Ub), mutant ubiquitin that cannot form K63 linkages (Ub(K63R), mutant ubiquitin that can only form K63 linkages (Ub(K63) and mutant ubiquitin that is deficient in forming K48 linkages (Ub(K48R)) and with or without (control) MIB2 followed by determination of cell surface $GABA_B$ receptors using $GABA_{B1}$ antibodies. Left, representative images (scale bar: 5 µm). Right, quantification of fluorescence intensities. The data represent the mean ± SEM of 20-22 neurons for each condition derived from two independent experiments. ns p>0.05; ** p< 0.005; *** p< 0.0005, two-tailed unpaired t-test.

FIGURE 8. **MIB2 mediates K63-linked ubiquitination of $GABA_B$ receptors.**
A, $GABA_{B1a}$(K->R) mutants, which are partially resistant to K63-linked ubiquitination, are not affected by overexpression of MIB2. Neurons were transfected with HA-tagged wild type $GABA_{B1}$ (WT $GABA_{B1}$), $GABA_{B1}$(K697/698), $GABA_{B1}$(K892R) or $GABA_{B1}$(K960R) with (+MIB2) or without (control) MIB2 and analyzed for cell surface expression of wild type and mutant $GABA_{B1}$ using HA antibodies. Left, representative images, scale bar: 5 µm. Right, quantification of fluorescence intensities. The data represent the mean ± SEM of 19-24 neurons per experimental condition derived from two independent experiments. ns p>0.05; ** p< 0.005, two-tailed unpaired t-test.

FIGURE 9. **$GABA_{B1a}$(K->R) mutants are resistant to glutamate-induced downregulation.**
Neurons transfected with HA-tagged wild type $GABA_{B1a}$ or HA-tagged $GABA_{B1a}$(K->R) mutants along with $GABA_{B2}$ were incubated in the presence (glutamate) or absence (control) of 50 µM glutamate for 90 minutes followed by cell surface staining for transfected $GABA_{B1}$ using HA antibodies. Left,

representative images, scale bar: 5 μm. Right, quantification of fluorescence intensities. The fluorescence intensity of neurons not treated with glutamate was set to 100%. The data represent the mean ± SEM of 20-25 neurons per experimental condition derived from two independent experiments. *** p< 0.0005, two-tailed unpaired t-test.

FIGURE 10. **Glutamate-induced downregulation of $GABA_B$ receptors is mediated by K63-linked ubiquitination.**
A, Sustained activation of glutamate receptors enhanced K63-linked ubiquitination of $GABA_B$ receptors. Neurons were incubated for 60 min in the absence (control) or presence of 50 μM glutamate and analyzed for K63-linked ubiquitination by *in situ* PLA using antibodies directed against $GABA_{B1}$ and K63-linked ubiquitin (white dots in representative images, scale bar: 5 μm). The graph depicts quantification of the *in situ* PLA signals. The data represent the mean ± SEM of 20 neurons derived from two independent experiments. ** p< 0.01; one way ANOVA, Dunnett's Multiple Comparison test.
B, Preventing K63-linked ubiquitination rendered $GABA_B$ receptors resistant to glutamate-induced downregulation. Neurons were transfected with wild-type ubiquitin (Ub(WT)), and mutants of ubiquitin that either permits only K63-linked ubiquitination (Ub(K63)) or prevents any kind of ubiquitin chain generation (Ub(KO)). Neurons were incubated for 90 min in the absence (control) or presence of 50 μM glutamate followed by determination of cell surface $GABA_B$ receptors using $GABA_{B1}$ antibodies. Left, representative images, scale bar: 5 μm. Right, quantification of fluorescence intensities. The data represent the mean ± SEM of 30-36 neurons from three independent experiments. *** p< 0.0001; two-tailed unpaired t-test.

FIGURE 11. Glutamate exposure increases the expression level of MIB2 and the MIB2/$GABA_B$ receptor interaction.
A, Increased MIB2 expression after glutamate exposure. Neurons were treated either for 15 min or 30 min with glutamate and analyzed for MIB2 expression. Left, representative images, scale bar: 10 μm. Right, quantification of fluorescence intensities. The data represent the mean ± SEM of 30 neurons from two independent experiments. *** p< 0.0001; one way ANOVA, Dunnett's Multiple Comparison test.
B, Increased interaction of $GABA_B$ receptors with MIB2 after glutamate exposure. Neurons were treated either for 15 min or 30 min with glutamate and analyzed for the interaction of MIB2 with $GABA_B$ receptors using *in situ* PLA. Left, representative images, scale bar: 5 μm. Right, quantification of the *in situ* PLA signals. The data represent the mean ± SEM of 14 neurons from two independent experiments. *** p< 0.0003; one way ANOVA, Dunnett's Multiple Comparison test.

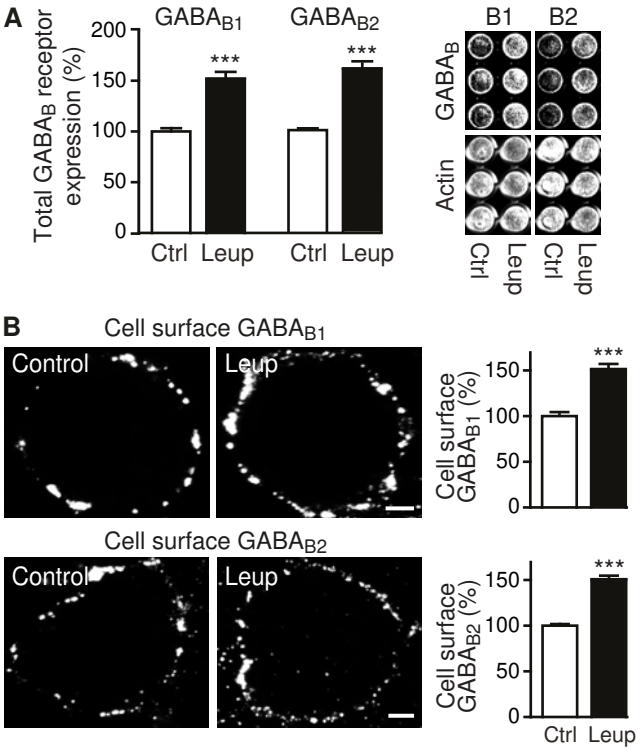

Fig. 1

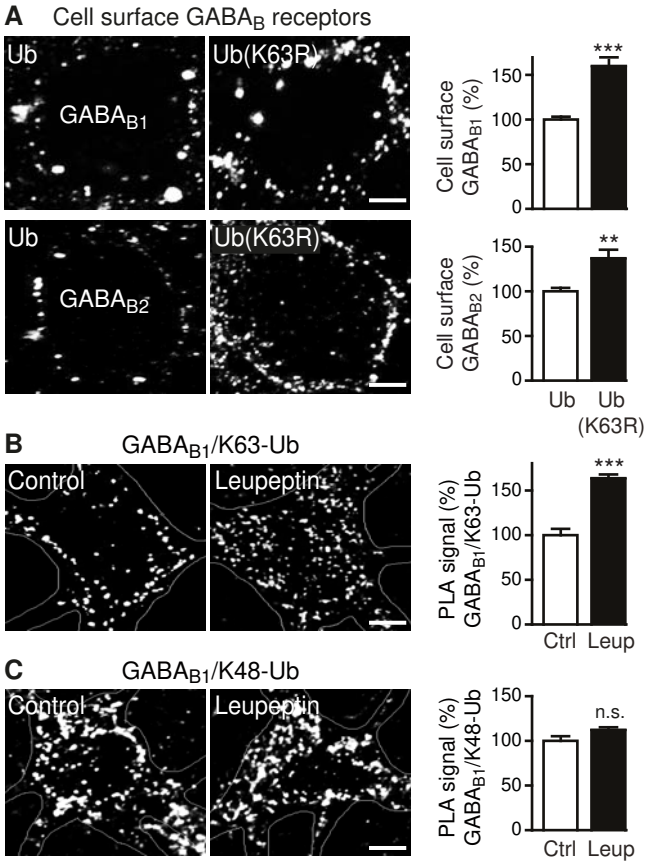

Fig. 2

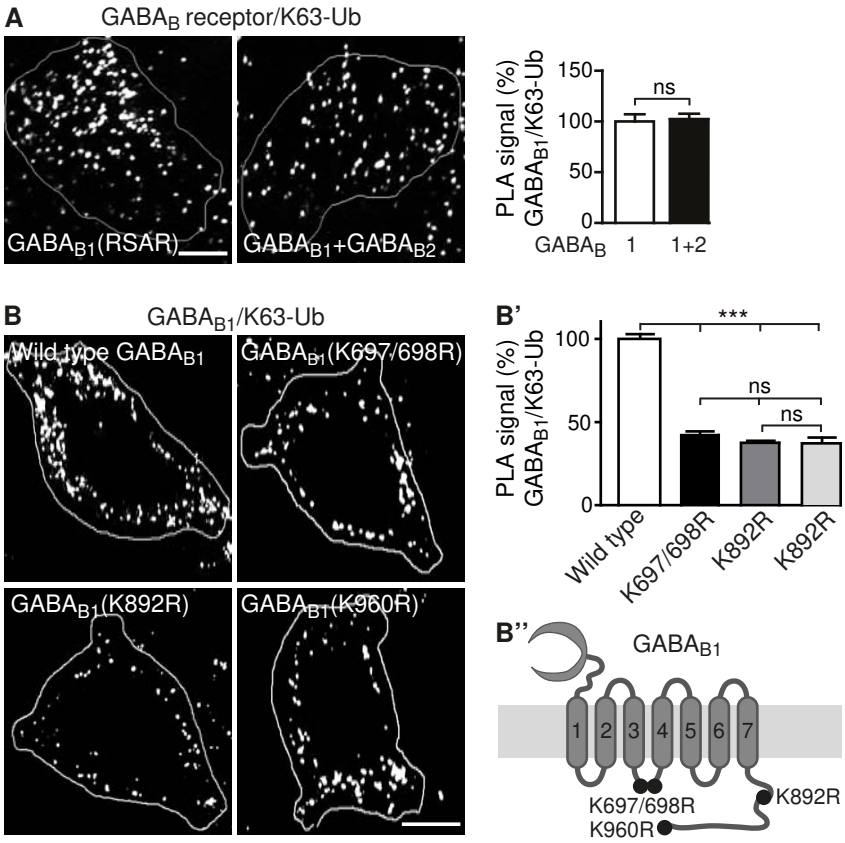

Fig. 3

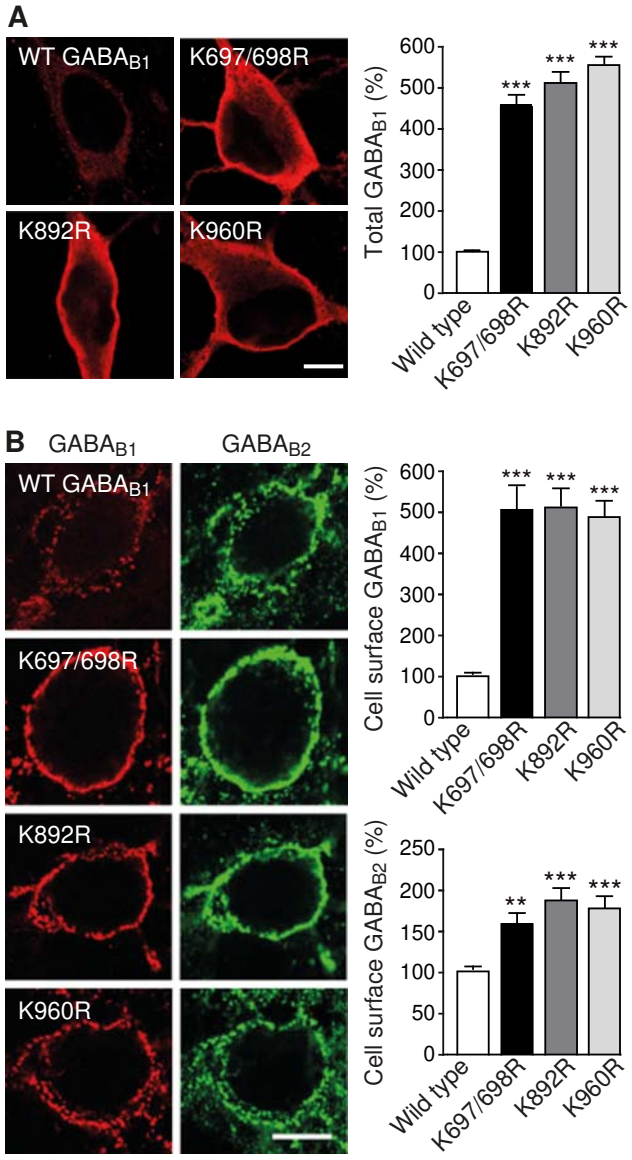

Fig. 4

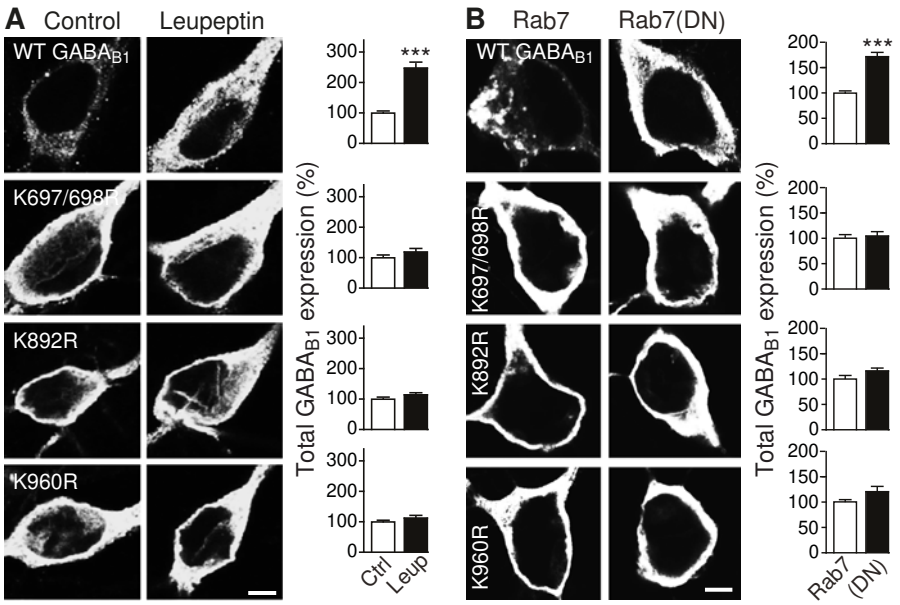

Fig. 5

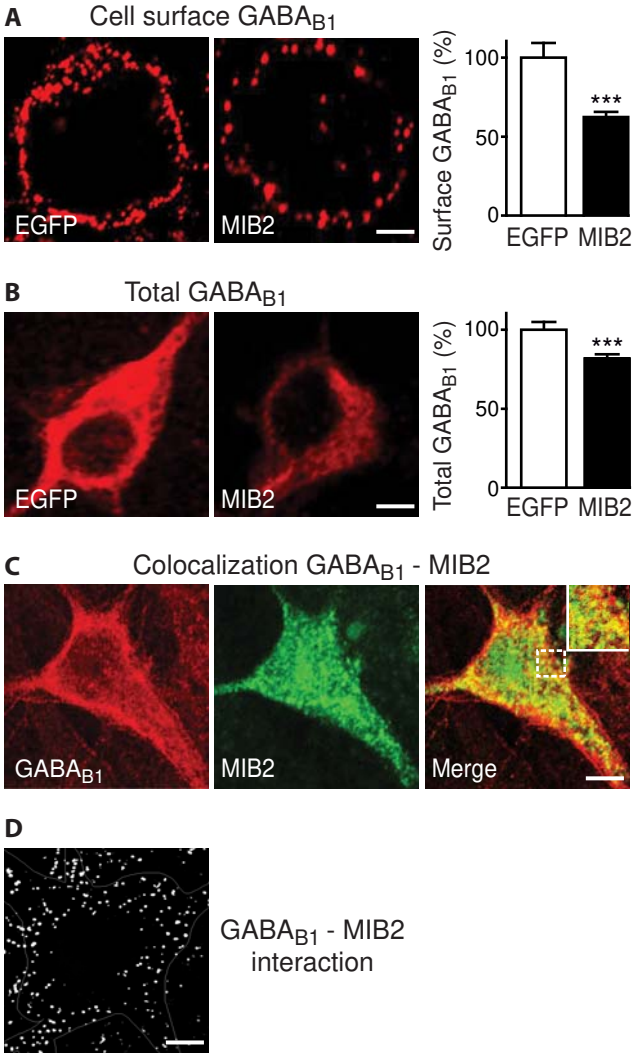

Fig. 6

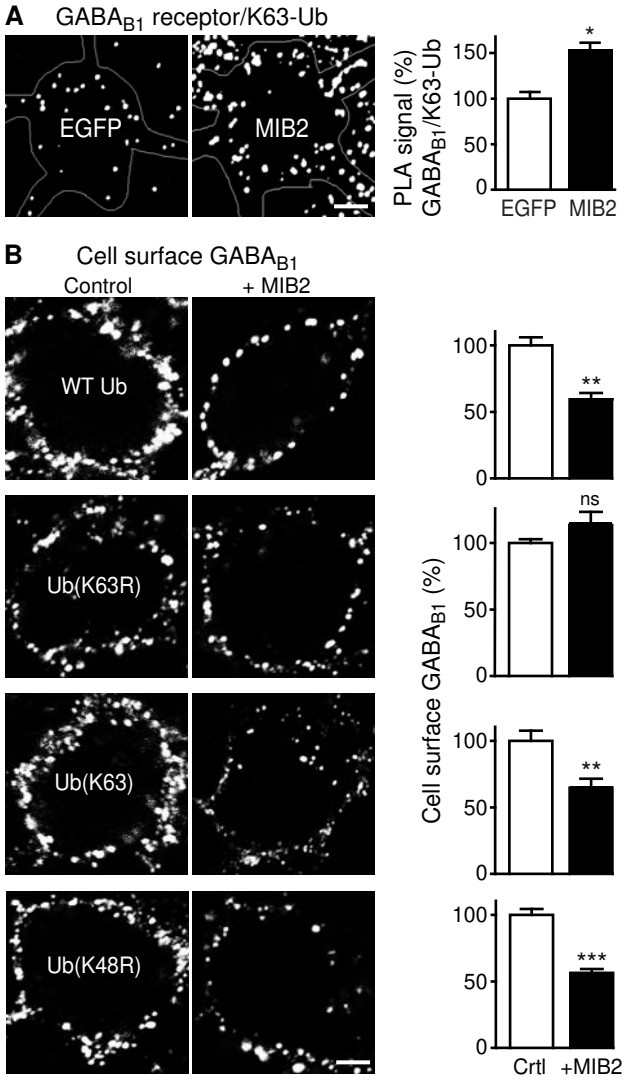

Fig. 7

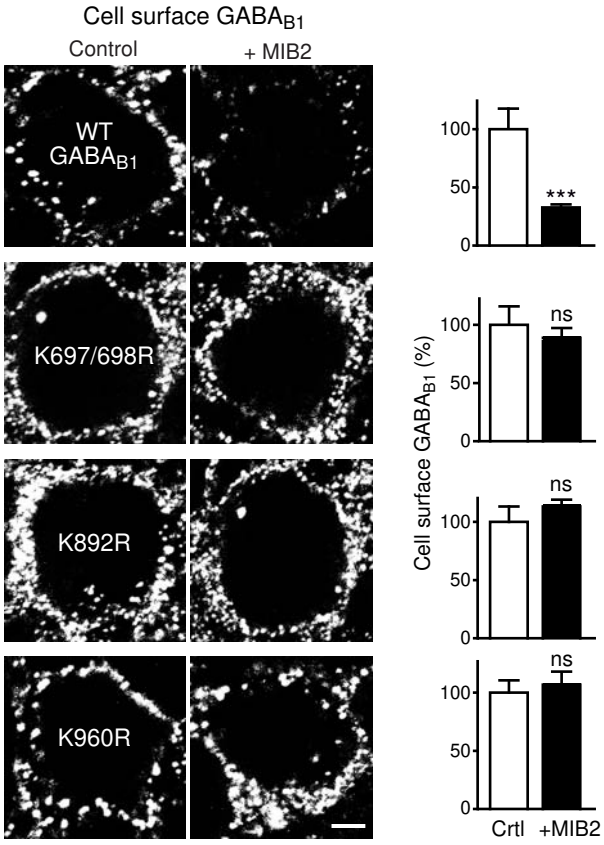

Fig. 8

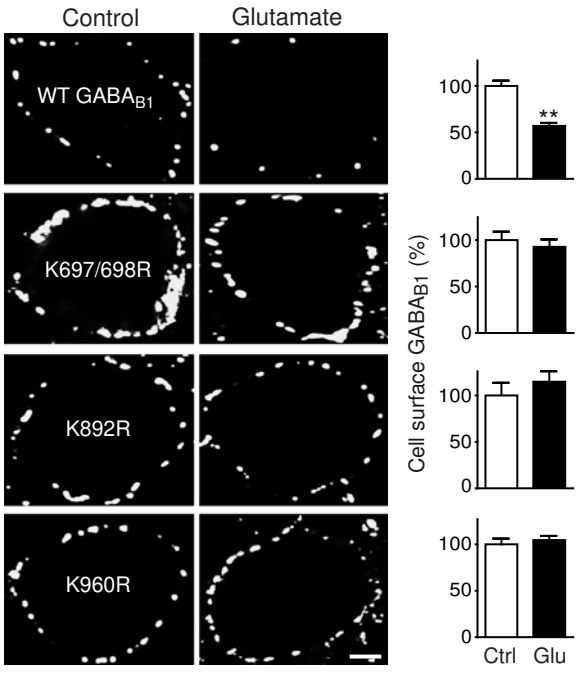

Fig. 9

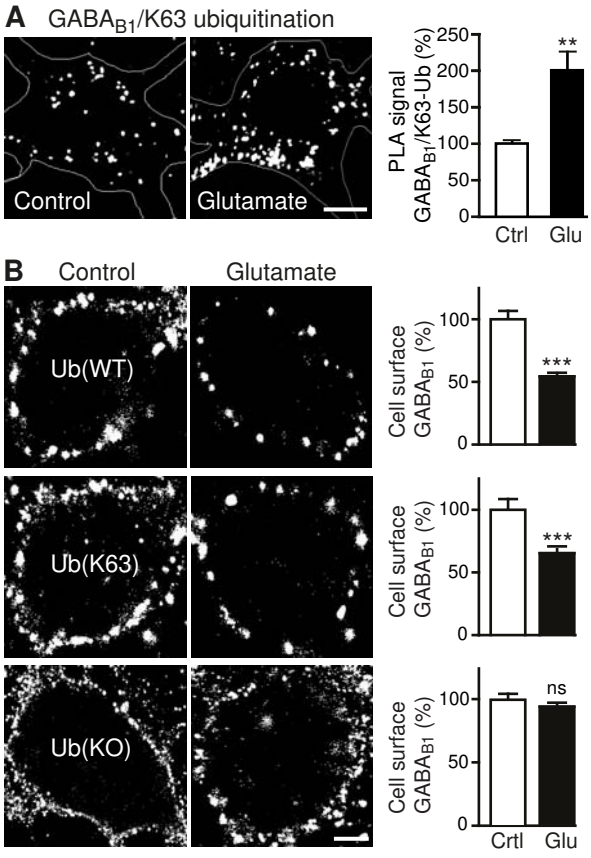

Fig. 10

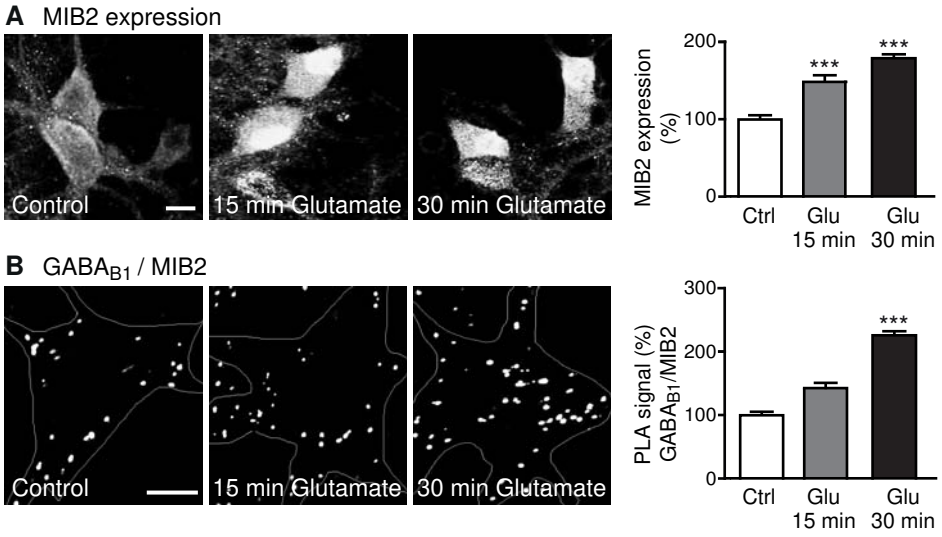

Fig. 11

K63-Linked Ubiquitination of $GABA_{B1}$ at Multiple Sites by the E3 Ligase Mind Bomb-2 Targets $GABA_B$ Receptors to Lysosomal Degradation

Khaled Zemoura, Claudia Trümpler and Dietmar Benke

J. Biol. Chem. published online August 29, 2016

Access the most updated version of this article at doi: 10.1074/jbc.M116.750968